A preliminary study of the role of bacterial–fungal co-inoculation on heavy metal phytotoxicity in serpentine soil

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Abstract. This study was conducted to understand the role of bacterial–fungal interactions on heavy metal uptake by \textit{Zea mays} plants. A pot experiment was conducted for 90 days with \textit{Z. mays} in serpentine soil inoculated with a Gram-negative bacterium, fungus (\textit{Aspergillus} sp.) and both microbes to determine the effects of inoculation on nickel, manganese, chromium and cobalt concentrations in plant tissue and soil. Soil nutrients and soil enzyme activities were measured to determine the effect of inoculations on soil quality. Inoculation of microorganisms increased shoot and root biomass, and the maximum biomass was in the bacterial–fungal inoculation. This could be due to the solubilisation of phosphate and production of indole acetic acid. Although the combination treatment contributed to an increase in heavy metal uptake in \textit{Z. mays} plants, the lowest translocation was observed in the combination treatment. Moreover, the soil available nitrogen, available phosphorous and total organic carbon content were increased with the microbial inoculation. Similarly, the soil dehydrogenase activity was higher as a result of microbial inoculation, whereas the highest dehydrogenase activity was reported in the combination inoculation. This study confirms the synergistic effect of bacterial–fungal inoculation as a soil-quality enhancer and as a plant-growth promoter in the presence of heavy metals.

Additional keywords: bioremediation, enzyme activity, heavy metal availability, soil quality, synergistic effect.

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Introduction

Weathering of ultramafic rocks produces soils and sediments that are non-anthropogenic sources of metal contamination. These soils are generally nutrient poor and contain high magnesium (Mg), iron (Fe), nickel (Ni), chromium (Cr) and cobalt (Co) and a high magnesium : calcium (Mg : Ca) ratio (Alexander 1988; Wenzel et al. 2003). In addition to the phytotoxic heavy metals, the high concentrations of Mg in soil can restrict Ca uptake, a limiting factor in plant tolerance to serpentine soils (Brady et al. 2005). Because of generally low organic matter and clay content (Proctor and Woodell 1975), serpentine soils often have a low water-holding capacity. Because of the high heavy metal concentrations, the microbial diversity is often low compared with non-serpentine soils (Panaccione et al. 2001; Southworth et al. 2014). Infertility, metal toxicity, and often sandy, rocky and shallow soils combined with low microbial diversity contribute to unique plant communities consisting of many rare and endemic species (Harrison and Rajakaruna 2011). Only well adapted species are able to tolerate the harsh chemical, physical and biological properties characteristic of serpentine soils (Anacker 2014).

The elevated metal concentrations associated with serpentinite rocks may cause ground-water pollution (Rajapaksha et al. 2012; Vithanage et al. 2014) and human and animal toxicities through plant uptake and food webs (Miranda et al. 2009). The presence of high concentrations of toxic metals can cause serious limitations to the use of areas overlaying serpentinites for agriculture and livestock farming (Shallari et al. 1998; Miranda et al. 2009). Geochemical studies from an agricultural area in Mouriki–Thiva in central Greece have revealed anomalous values of Ni (621–2639 mg kg\textsuperscript{-1}) and Cr (134–856 mg kg\textsuperscript{-1}), where Cr and Ni are primarily mobilised from chromite, olivine and serpentine minerals (Antibachi et al. 2012). Ni is substantially more labile, and, as a result, is readily available to plants in high concentrations (Antibachi et al. 2012; Vithanage et al. 2014). Ni is a known neurotoxin, reproductive toxin, nephrotoxin, hepatotoxin and a
carcinogenic agent (Denkhaus and Salnikow 2002). Cr is also a potent carcinogenic agent (Dayan and Paine 2001). Exposure to manganese (Mn) through drinking water can result in permanent neurological disorders and cardiac, liver, reproductive and fetal toxicities (Crossgroe and Zheng 2004).

The efficiency and the type of metal uptake depend not only on the species of plant but also the action of rhizospheric organisms (Abou-Shanab et al. 2003; Ma et al. 2009). The rhizosphere is a complex and dynamic environment that involves many physical and chemical reactions (Jones and Darrah 1994; Anjum et al. 2012; Neilson and Rajakaruna 2012). In recent years, the role of rhizobacteria on plant heavy metal uptake has received some attention (Burd et al. 1998; Burd et al. 2000; Rajkumar and Freitas 2008). Many rhizospheric bacteria have the ability to promote plant growth through various mechanisms, including nitrogen (N) fixation, utilisation of 1-aminoacyclopropane-1-carboxylic acid (ACC) and production of siderosphore and plant-growth regulators (Burd et al. 1998; Ma et al. 2009). These mechanisms increase the plant biomass and tolerance to heavy metal toxicity. Even though several studies have been conducted on the influence of bacteria in plant heavy metal uptake and immobilisation (Ma et al. 2009), there are no published reports of bacterial–fungal interactions on plant heavy metal uptake. Bacterial–fungal interactions are more apparent as biofertilisers in the form of biofilm (Seneviratne et al. 2009) and have shown their potential to be used in waste-water reactors for heavy metal remediation (Herath et al. 2013). Studies have also shown that their performance is higher than that of mono- or mixed cultures of bacterial biofilms (Herath et al. 2013). Thus, the present study was conducted to examine the role of bacterial–fungal inoculation on Ni, Mn, Cr and Co uptake on Zea mays plants grown in serpentine soils.

**Materials and methods**

**Study site**

Serpentine soil samples were collected from the Yudhaganawa serpentine site located within the Wasgamuwa National Park (7°11′67″N, 80°93′33″E) in the Matale and Polonnaruwa districts of north-central Sri Lanka (Vithanage et al. 2014), found in a transitional zone between the Highland and the Vijayan Complex. The climate is tropical with a dry period of 8–9 months. Rainfall is from the north-eastern monsoon from October to January and mean temperature is uniformly high at 32°C throughout the year. Mean annual rainfall ranges from 1750 mm to 2250 mm. The vegetation is mostly a dry mixed evergreen forest (57%) and a scrub jungle (27%).

**Soil collection**

Soil samples were collected within 10–15 cm from the surface after clearing the surface litter from five random locations. The samples were sealed in polythene bags and brought immediately to the laboratory and bulked and mixed together. The initial metal concentrations were 6567, 2609, 14,880 and 555 mg kg⁻¹ of Ni, Mn, Cr and Co, respectively (Vithanage et al. 2014).

**Preparation of microbial inoculums**

Heavy metal-resistant bacteria were isolated in nutrient agar (NA) from serpentine soil collected from the serpentinite outcrop at Yudhaganawa. Dilution plating with serpentine soil from Yudhaganawa (10⁻¹–10⁻³) was carried out to isolate the heavy metal-resistant bacteria present in serpentine soil. Fungal–bacterial biofilms were formed with an Aspergillus fungus (known for metal-tolerant strains; Ahmad et al. 2006; Anahid et al. 2011). The biofilms were subjected to a series of Ni concentrations (50–500 ppm) and the adsorption was determined. The biofilm with the highest adsorbing ability was used in the experiment.

The Gram-negative bacterium isolated from Ni-rich serpentine soil (currently, unidentified), a garden soil species of Aspergillus, and both bacteria and fungi were used as inoculums in the study. The bacterial cells were grown overnight in 250-mL Erlenmeyer flasks containing 100 mL of sterilised nutrient broth on a rotary shaker at 100 rpm at 30°C until late log phase. The fungus was cultured in 250-mL Erlenmeyer flasks containing 100 mL of Czapek dox broth in a rotary shaker at 100 rpm at 30°C for 48 h.

**Glasshouse experiment**

Serpentine soil was collected from Yudhaganawa outcrop and sieved to obtain the <2 mm fraction. The soils were inoculated with bacteria (B) (10 mL from the bradyrhizobium culture of 0.517 optical density at 600 nm), fungi (F) (10 mL of fungal broth culture containing 2 g of fungal mycelium) and bacteria and fungi together (BF), in triplicate (3 pots, 25 × 20 × 10 in size, per inoculum treatment). The control was filled with serpentine soil, without any microbial treatment. Zea mays was selected because it has the ability to tolerate heavy metal stress (Hall 2002; Nocito et al. 2006). Surface-sterilised Z. mays seeds were soaked in water overnight and allowed to germinate in a Petri dish lined with filter paper. After 1 week, three seedlings of equal height were planted in each pot. Plants were allowed to grow for 90 days in a glasshouse at 26–30°C and 70% relative humidity, with 12 h light/12 h dark conditions (natural light). Pots were watered periodically to keep the soils moist.

**Plant tissue analysis**

After 90 days, Z. mays plants were uprooted, washed with deionised water, and shoot and root samples were separated and dried at 50°C. Dried plant samples were weighed and digested with concentrated HNO₃ acid in a close-vessel temperature-controlled microwave digester system (Milestone ETHOS PLUS labstation with HRP-1000/10S high-pressure segmented rotor, Milestone Model START D, Italy). The digest was diluted to 100 mL with deionised water and Ni, Mn, Cr and Co concentrations were determined using an atomic adsorption spectrophotometer (GBC 933 M, Melbourne, Vic., Australia). Plant accumulation factor and translocation factor were calculated using the following equations:

\[
\text{Plant accumulation factor} = \frac{\text{metal concentration in root}}{\text{metal concentration in soil}}, \quad \text{and} \quad (1)
\]

\[
\text{Translocation factor} = \frac{\text{metal concentration in shoot}}{\text{metal concentration in root}}. \quad (2)
\]

**Analysis of soil nutrients**

Available phosphorus (P) was measured by the sodium bicarbonate extraction method. About 1.25 g of fresh soil was
shaken at 180 rpm with 25 mL of 0.5 M sodium bicarbonate for 15 min. The extract was filtered with Whatman No. 42 filter paper and 1 mL of it was used for analysis. To the filtrate, 4 mL of ascorbic acid and 3 mL of molybdate reagent were added and, after 1 h, absorbance was read at 880 nm (Watanabe and Olsen 1965). Available N was measured using the colourimetric method (Cataldo et al. 1975). A sample of 10 g of soil was shaken with 20 mL of K2SO4 for 30 min at 60 rpm. An aliquot of 0.5 mL of the extract was mixed with 1.0 mL of salicylic acid and mixed well with a vortex mixture. After 10 min, 10 mL of sodium hydroxide was added and mixed well. The mixture was incubated for 1 h for colour development and absorbance was read using a spectrophotometer (Shimadzu UV-2450, Japan) at 410 nm.

Measuring soil enzyme activities

To measure the polyphenol oxidase activity, ~5 g of soil was mixed with 10 mL H2O, 6 mL 0.1% ascorbic acid and 10 mL 30% triphenyltetrazolium chloride (TTC) was added to 6 g of the soil and mixed well with a vortex mixture. After 15 min, 10 mL of 0.5 mL of the extract was mixed with 1.0 mL of salicylic acid and with continuous agitation. Then 3 mL aliquot of 30% H2O2 (pH 2 with HNO3) was added and the sample was heated again to 85°C for 3 h, with intermittent agitation. After cooling, 5 mL of 3.2 M NH4OAc in 20% (v/v) HNO3 was added and the sample was diluted to 20 mL and agitated continuously.

Statistical analysis

Data were analysed by ANOVA in SAS statistical package (version 9.1, Statistical Analysis System Institute Inc, NC, USA). Means were compared using Duncan’s multiple-range test (DNMRT) at P = 0.05.

Results

Plant dry weight and height

Both shoot and root dry weights were higher in plants with microbial inoculation than in the control treatment (Table 1). Root dry weight showed a significant increase in B and BF treatments. The maximum root dry weight was recorded from BF treatment, which was 160% higher than that in the control. The shoot dry weight of microorganism-inoculated samples showed a significant increase over the control. The highest shoot dry weight was recorded for the F treatment. It was 73% higher than in the control. Both of the other microbial treatments (B and BF) showed a 63% increase of shoot dry weight over the control. There was a significant difference in shoot lengths among the treatments, with the highest shoot length reported in the F treatment, showing an increase of 29% compared with the control.

Even though not significant, the highest root length was observed in the BF treatment, with a 32% increase over the control.

Heavy metal accumulation in plant roots and shoots

Plant roots, irrespective of treatments, absorbed Ni in higher concentrations than other metals (Fig. 1). Shoot samples showed significantly higher concentrations of both Ni and Mn than Cr and Co, irrespective of the treatments. Plants treated with BF increased their uptake by 13% for Ni, 52% for Mn, 83% for Cr and 56% for Mn compared with control plants where no inoculum was added (Fig. 1). Even though it was not significant, the translocation factor (Table 2) was lowest in the BF treatment, for Ni and Mn, whereas it was second-lowest for Cr and not detected for Co. Among the heavy metals tested, the highest translocation factor was observed for Mn, suggesting higher accumulation in shoots. Although not significant, the plant accumulation factor was higher in the BF treatment than in the other treatments (Table 2). This indicated that the presence of microbes increased the heavy metal bioavailability and decreased translocation of Ni, Mn and Cr.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Root weight (g)</th>
<th>Shoot weight (g)</th>
<th>Root length (cm)</th>
<th>Shoot length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.05 ± (0.02)</td>
<td>0.19 ± (0.06)</td>
<td>14.6 ± (5.34)</td>
<td>9.6 ± (1.08)</td>
</tr>
<tr>
<td>Bacteria</td>
<td>0.09 ± (0.03)</td>
<td>0.31 ± (0.07)</td>
<td>18.8 ± (7.91)</td>
<td>12.1 ± (1.24)</td>
</tr>
<tr>
<td>Fungi</td>
<td>0.06 ± (0.02)</td>
<td>0.33 ± (0.08)</td>
<td>15.2 ± (4.54)</td>
<td>12.7 ± (0.97)</td>
</tr>
<tr>
<td>Bacterial–fungal inoculation</td>
<td>0.13 ± (0.03)</td>
<td>0.31 ± (0.07)</td>
<td>18.9 ± (3.29)</td>
<td>12.2 ± (1.47)</td>
</tr>
</tbody>
</table>

Table 1. Shoot and root lengths of Zea mays plants in different treatments

Values in parentheses represent standard deviation.
Soil nutrients

The total organic carbon (TOC) content was 2.3% in the serpentine soil from Yudhaganawa. After inoculation of microbes, F treatment showed a significant increase in TOC content, which showed a 15% increase over the control (Table 3). The B and BF treatments showed a significant reduction in TOC over the control by ~7.5% and 5%, respectively. Moreover, the inoculation of microbes led to an increase in both available N and P. Even though there was no significant difference in available N content among the treatments, the highest value was obtained in B treatment and the available P content was significantly higher in the BF treatment.

Soil enzyme activities

The dehydrogenase activity did not show a significant difference among the treatments. However, it was highest in the BF treatment. The polyphenol oxidase activity was significantly higher in the BF treatment, whereas, the catalase activity was significantly lower in the control (Fig. 2).

Fractionation of heavy metals

The sequential extraction results revealed that the inoculation of microbes into soil does not show significant differences of fractionation in Ni, Mn, Cr and Co among the treatments. However, exchangeable fractions of Ni and Mn were higher (132 and 47 mg kg⁻¹, respectively) in the B treatment than in the other treatments. Carbonate-bound fraction was higher in the BF treatment for Ni, Mn and Co than in the other treatments. Similarly, both Fe–Mn-bound and organic matter-bound fractions were higher in the BF treatment (Fig. 3).

Discussion

Even though heavy metals are toxic to living cells, the microorganisms that inhabit heavy metal-contaminated areas are resistant to metal toxicity (Haferburg and Kothe 2007; Gadd 2010). Therefore, several studies have focussed on the application of these microbes in bioremediation (Burd et al. 1998;
Rajkumar and Freitas (2008). Belimov et al. (2005) reported that the inoculation of the cadmium (Cd)-resistant bacterial strains isolated from the rooting zone of Indian mustard (*Brassica juncea*) grown in Cd-contaminated areas enhances its growth under toxic heavy metal concentrations. Similarly, Jiang et al. (2008) reported that the inoculation of *Burkholderia* sp. isolated from a lead (Pb)- and Cd-contaminated field enhanced the growth of tomato and maize under Pd and Cd stress. Serpentine soil is a naturally metal-contaminated soil and, therefore, the microbes that live in this habitat are likely to be more resistant to heavy metal concentrations. 

![Fig. 2](image-url)  
Fig. 2. (a) Catalase, (b) polyphenol oxidase and (c) dehydrogenase activity of serpentine soil inoculated with bacteria (B), fungi (F) and both bacteria and fungi (BF). Control soil (C) was provided with no inoculum. Different letters indicate significant differences (*P* = 0.05, d.f. = 8). Error bars represent the standard error of the mean.

![Fig. 3](image-url)  
Fig. 3. (a) Exchangeable fraction, (b) carbonate bound fraction, iron (Fe)–manganese (Mn)-bound fraction of nickel (Ni), manganese (Mn), chromium (Cr) and cobalt (Co) in serpentine soil inoculated with bacteria (B), fungi (F) and both bacteria and fungi (BF). Control soil (C) provided with no inoculum. Different letters indicate significant differences (*P* = 0.05, d.f. = 8). Error bars represent the standard error of the mean.
metals than those in more recently contaminated areas. (Ma et al. 2014) reported that Psychrobacter sp. and Pseudomonas sp. isolated from serpentine soil improved the growth of B. juncea and Rícinusí communis grown in serpentine soil.

In the present study, the bacterial strains were isolated from serpentine soils with an objective to assess the effects of the metal-resistant and plant growth-promoting bacteria (PGPB) on plant growth and uptake of Ni, Mn, Cr and Co by Z. mays. The study showed that the inoculation of microorganisms increased the growth of Z. mays and was also effective in protecting plants from growth inhibition caused by heavy metals. Both shoot and root dry weight and shoot length and root length were higher in microorganism-inoculated samples than in the control. Similarly, a significant increase in shoot and root was observed with the inoculation of Methylobacterium oryzae strain and Burkholderia sp. into tomato plants grown under Ni and Cd stress (Madhaiyan et al. 2007). In our study, the highest root weight was observed in the bacterial–fungal treatment (BF), which was 2.6 times higher than in the control. This may be due to the secretion of plant growth hormones (IAA) by the synergistic effect of the bacterial–fungal interaction (Glick 2012).

Heavy metal-tolerant bacteria in the rhizosphere play an import role in growth promotion by possessing many different mechanisms, such as siderophore production, utilisation of siderophores (Burd et al. 1998, 2000). Even though there are several studies on bacterial influence on heavy metal uptake in soil, very few have focused on fungi. However, studies have reported the effect of mycorrhizal fungi on heavy metal uptake: uptake by mycorrhizal fungi depends on plant growth conditions, the fungal partner, heavy metal and amount of metal present in soil (Weissenhorn et al. 1995; Southworth et al. 2014).

The plant accumulation factor was highest with Ni, followed by Mn, showing the favourability of Ni and Mn uptake over other metals (Table 3). The plant accumulation factor was lowest with Cr and this could be due to the toxic nature of Cr. It is reported that Mn is a readily translocatable metal, whereas Ni is intermediate and Cr is categorised as the least translocatable metal (Alloway 1995). The amount of heavy metal translocation is a critical consideration for both phytoremediation and vegetative consumption. Higher translocation is favourable in phytoremediation processes, whereas it is less desirable in edible plants used for consumption. In the present study, the translocation of Ni, Mn, Cr and Co was lowest in the BF treatment, showing the lowest accumulation in shoots. Translocation factor, the ratio of shoot to root for metals, indicates internal metal transportation (Kabata-Pendias 2010). It is mainly dependent on heavy metal mobility and toxicity. We report the maximum translocation for Mn, a micronutrient, likely explaining the higher translocation we observed. Ni, which also showed considerable translocation, is also reported as a plant micronutrient important for growth and metabolism (Mishra and Kar 1974; Brown et al. 1987; Barker and Pilbeam 2014). Our results indicated that metals accumulated by Z. maise were largely retained in roots, as shown by values of translocation factor of <1.

The F-treatment was more effective than the other treatments with increasing TOC. The secretion of mucilage/polysaccharides by inoculated fungi could be the reason for the increase in the TOC concentration (Srivastava et al. 2012). The content of available N did not show a significant increase or decrease with the introduction of microbes. However, the P availability was higher in the B and BF treatments. Soil microorganisms produce a range of phosphatases, which have the capacity to utilise P from various forms of organic P that occur in soil. Enhanced phytase activity in the rhizosphere is responsible for P deficiency across a wide range of plant species and is commonly reported to be higher in P-deficient soils (Richardson and Simpson 2011). A wide range of microorganisms able to solubilise inorganic P have been cultured from soil, including bacteria (e.g. Actinomycetes, Pseudomonas and Bacillus spp.) and fungi (e.g. Aspergillus and Penicillium spp.) (Richardson and Simpson 2011).

Soil enzyme activities are directly related to soil physiochemical characteristics, soil microbial diversity, and soil nutrients (Caldwell 2005). Among the different soil enzyme activities, dehydrogenase activity is an indicator for potential non-specific intracellular enzyme activity of the total microbial biomass (Ladd 1978; Chu et al. 2007). In the present study, the microorganism-inoculated samples showed a higher
Microbial interactions on plant metal uptake

**References**


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